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Associations between Blood and Urine Arsenic Concentrations and Global Levels of Post-Translational Histone Modifications in Bangladeshi Men and Women

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Abstract

Background: Exposure to inorganic arsenic is associated with numerous adverse health outcomes, with susceptibility differing by sex. While evidence from *in vitro* studies suggests that arsenic alters post-translational histone modifications (PTHMs), evidence in humans is limited.

Objectives: The objectives were to determine: 1) if arsenic exposure is associated with global (%) levels of PTHMs: H3K36me2, H3K36me3, and H3K79me2 in a sex-dependent manner and 2) if %PTHMs are stable when arsenic exposure is reduced.

Methods: We examined associations between arsenic, measured in blood and urine, and %PTHMs in peripheral blood mononuclear cells from 317 participants enrolled in the Bangladesh Folic Acid and Creatine Trial (FACT). We also examined the stability of %PTHMs after the use of arsenic-removal water filters (n = 60).

Results: Associations between natural log-transformed (ln) urinary arsenic, adjusted for creatinine (uAs_{Cr}), and %H3K36me2 differed significantly between men and women ($p = 0.01$). Ln-uAs_{Cr} was positively associated with %H3K36me2 in men (β = 0.12; 95% CI: 0.01, 0.23, $p = 0.03$), but was negatively associated with %H3K36me2 in women (β = -0.05; 95% CI: -0.12, 0.02, $p = 0.19$). The patterns of associations with blood arsenic were similar. On average, water filter use was also associated with reductions in %H3K36me2 ($p < 0.01$), but this did not differ significantly by sex. Arsenic was not significantly associated with %H3K36me3 or %H3K79me2 in men or women.

Conclusions: Arsenic exposure was associated with %H3K36me2 in a sex-specific manner, but was not associated with %H3K36me3 or %H3K79me2. Additional studies are needed to assess changes in %H3K36me2 after arsenic removal.

Introduction

Worldwide, more than 140 million people are exposed to arsenic-contaminated drinking water (Bagchi 2007); in Bangladesh alone, up to 57 million individuals are exposed (Kinniburgh et al. 2003). Chronic exposure to arsenic causes bladder, lung, and skin cancers and is also associated with numerous non-cancer health outcomes (NRC 2013). Susceptibility to many of these arsenic-related health outcomes differs by sex, with some outcomes preferentially afflicting males and others females (NRC 2013). For example, males are more likely to develop arsenic-induced skin lesions (Ahsan et al. 2006b; Watanabe et al. 2001) and skin, liver, and bladder cancers (Chen and Wang 1990; Chen et al. 2003; Leonardi et al. 2012), while females may be more susceptible to arsenic-induced developmental outcomes (Gardner et al. 2013; Hamadani et al. 2011; Saha et al. 2012) and cardiovascular disease (CVD) (Moon et al. 2013). However, the mechanisms underlying these sex differences remain unknown.

Experimental studies and observational studies in human populations have demonstrated that arsenic alters epigenetic modifications, including global 5-methylcytosine (5-mC) (Niedzwiecki et al. 2013; Pilsner et al. 2007; Ren et al. 2010; Tellez-Plaza et al. 2014) and 5-hydroxymethylcytosine (5-hmC) (Niedzwiecki et al. 2015; Zhang J et al. 2014), and there is evidence that these effects differ by sex (Broberg et al. 2014; Niedzwiecki et al. 2015; Nohara et al. 2011; Pilsner et al. 2012). *In vitro* and rodent studies have also shown that arsenic alters global (%) post-translational histone modifications (PTHMs) in tissues or cell lines derived from tissues that are targets of arsenic toxicity, such as the lung (Zhou et al. 2008), bladder (Chu et al. 2011), and brain (Cronican et al. 2013), and the effects of arsenic on %PTHMs in the brain have been shown to be sex-dependent in mice (Tyler et al. 2015). An epidemiological study of 63 male steel workers reported that arsenic exposure via inhalation was associated with higher

global levels of histone H3 lysine 4 dimethylation in white blood cells (WBCs) (Cantone et al. 2011); however, since this study only included men, potential differences by sex could not be examined.

In a previous study of 40 Bangladeshi adults, we observed sex-specific associations between arsenic, measured in urine, and several %PTHMs (H3 lysine 4 trimethylation, H3 lysine 27 trimethylation, and H3 lysine 27 acetylation) in peripheral blood mononuclear cells (PBMCs) (Chervona et al. 2012). We have also previously observed sex-specific associations between arsenic exposure and global levels of both 5-mC and 5-hmC in PBMC DNA (Niedzwiecki et al. 2015). Thus, we now present data on three PTHMs: histone H3 lysine 79 di-methylation (H3K79me2), selected because it has been shown to regulate the expression of Tet1 (Huang et al. 2013; Williams et al. 2011), which converts 5-mC to 5-hmC (Ito et al. 2011), and it is dysregulated in cancers (Bernt et al. 2011; Kim et al. 2012; Zhang L et al. 2014), and histone H3 lysine 36 di- and tri-methylation (H3K36me2 and H3K36me3, respectively), because these two PTHMs have been shown to be altered by arsenic *in vitro* (Zhou et al. 2008) and they are also dysregulated in several types of cancer (Duns et al. 2010; Fontebasso et al. 2013; He et al. 2011; Tamagawa et al. 2013). This study utilized a subset of PBMC samples collected from participants enrolled in the Folic Acid and Creatine Trial (FACT) (Peters et al. 2015). First we evaluated sex-specific associations between arsenic and our candidate PTHMs using baseline FACT samples. Then, since all participants in the trial were provided with arsenic-removal water filters at enrollment, we evaluated whether %PTHMs were altered after reducing arsenic exposure; this was achieved using samples collected at baseline and week 12 from participants who did not receive a dietary supplement. The data reported herein add to a growing body of

evidence that arsenic induces epigenetic dysregulation on a global level and, moreover, that this often occurs in a sex-specific manner.

Study Participants and Methods

Region and Participants

In 2010, participants for the current study (FACT) were recruited from the Health Effects of Arsenic Longitudinal Study (HEALS), a prospective cohort study which initially recruited 11,746 adults (between the ages of 20 and 65) living in a 25 km^2 region in Araihazar, Bangladesh (Ahsan et al. 2006a). FACT participants were randomly selected from all HEALS participants who had been drinking from household wells with water arsenic ≥ 50 μg/L, the Bangladesh standard for safe drinking water. Exclusion criteria included: pregnancy, nutritional supplement use, and adverse health outcomes, including proteinuria, renal disease, diabetes, gastrointestinal disease, chronic obstructive pulmonary disease, skin lesions, and cancer. Informed consent was obtained by Bangladeshi field staff physicians. This study was approved by the Institutional Review Board of Columbia University Medical Center and the Bangladesh Medical Research Council.

Study Design

The FACT study is a randomized, placebo-controlled trial that had the primary goal of determining whether folic acid (FA) and/or creatine supplementation reduces blood arsenic (bAs) concentrations in arsenic-exposed, Bangladeshi adults (Peters et al. 2015). All FACT participants received an arsenic-removal water filter (READ-F filter, Brota Services International, Bangladesh) at baseline to be used for the duration of the study and thereafter. Participants (n = 622) were also randomized to one of five nutrition intervention treatment arms:

placebo (n =104), 400 µg FA/day (n = 156), 800 µg FA/day (n = 154), 3 g creatine/day (n = 104), and 3 g creatine + 400 µg FA/day (n = 104) (Peters et al. 2015). Whole blood and urine samples were collected from participants at baseline, week 12, and week 24; sample collection and handling have been described previously (Chervona et al. 2012; Peters et al. 2015). For the current study, we used histones isolated from baseline (i.e., pre-intervention)-collected PBMCs from a subset of FACT participants from all five treatment arms (see **Supplemental Material, Figure S1**), who had whole blood, urine, and PBMC samples and complete data for arsenic measures, %PTHMs, and potential confounders (n = 317). We also used all available PBMCs collected at baseline and week 12 from participants in the placebo group (n = 60) to examine if %PTHMs were altered after the use of arsenic-removal water filters; due to filter use, participants in the placebo group experienced a significant decrease in bAs concentrations from baseline to week 12 (Peters et al. 2015).

General Characteristics

General characteristics of the study participants were determined at baseline by an in-person questionnaire. Body mass index (BMI) was calculated from the measured weight and height of each participant (kg/m^2) at baseline.

Total Blood Arsenic

As described previously (Peters et al. 2015), total bAs concentrations were measured using a Perkin-Elmer Elan DRC II ICP-MS equipped with an AS10+ autosampler. The intra- and inter-assay CVs for bAs were 2.7% and 5.7%, respectively.

Total Urinary Arsenic

We measured total urinary arsenic (uAs) by graphite furnace atomic absorption spectrophotometry, using the AAnalyst 600 graphite furnace system (Perkin Elmer, Shelton, CT), based on a method by Nixon et al. (Nixon et al. 1991). Intra- and inter-assay CVs for uAs were 3.1% and 5.4%, respectively. These values were adjusted for urinary creatinine (uCr) concentrations, measured by a method based on the Jaffe reaction (Slot 1965). The intra- and inter-assay CVs for uCr were 1.3% and 2.9%, respectively.

Plasma Folate and B12

Plasma folate and B12 were measured by radio-protein-binding assay (SimulTRAC-SNB, MP Biomedicals). The intra- and inter-assay CVs were 5% and 13%, respectively, for folate and 6% and 17%, respectively for B12.

Histone Isolation

While we recently identified a cleavage product of histone H3 in human PBMCs that interferes with the measurement of PTHMs residing downstream of H3 cleavage sites (Howe and Gamble 2015), we note that the PTHMs in the current study are located upstream of cleavage sites and are therefore not impacted by this. Histones were isolated from PBMCs by acid extraction, as described previously (Chervona et al. 2012). Briefly, PBMCs were lysed in radioimmunoprecipitation assay buffer, supplemented with a protease inhibitor cocktail (Roche) and 1 μM of protease inhibitor E-64, for 10 min. The cell lysate was passed through a 21-gauge needle, and the pellet was collected by centrifugation, washed in histone washing buffer, collected again after centrifugation, and resuspended in 0.4 N H_2SO_4. After incubation at 4°C overnight, the supernatant was collected by centrifugation, mixed with acetone, and incubated

overnight at -20°C. Pellets were collected by centrifugation, washed with acetone, dried, and resuspended in 4 M urea. Histone concentrations were determined by the Bradford Assay, according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Samples were aliquoted and stored at -80°C.

%H3K36me2, %H3K36me3, %H3K79me2

%PTHMs were measured by sandwich ELISA, based on a previously described method (Chervona et al. 2012). Polystyrene 96-well microplates (Fisher Scientific) were coated with a capture antibody for total histone H3 (Abcam, 1:20,000) and incubated overnight at 4ºC. The next day, plates were blocked with 3% milk diluted in PBST (1 X PBS, 0.05% TWEEN-20) for 2 hours and then washed with PBST. Histone samples from FACT participants were diluted with ddH_2O. Sample dilutions for each assay were as follows: H3K36me2, 1 ng/µL; H3K36me3, 1.5 ng/µL; H3K79me2, 2.0 ng/ µL. A standard curve was made with mixed histones from calf thymus (Sigma), and a pooled blood sample was included on each plate for calculating inter-assay CVs. FACT histone samples, calf histones, and the pooled blood sample were plated in duplicate. Plates were incubated on an orbital shaker at room temperature for 1.5 hours, then wells were washed with PBST. Detection antibodies were diluted in 1% milk PBST, to further prevent potential background signal, and 100 µL was added to each well of the appropriate plate. Detection antibody dilutions were as follows: total H3, Sigma, 1:40,000; H3K36me2, Abcam, 1:2,000; H3K36me3, Abcam, 1:2,000; H3K79me2, Active Motif, 1:1,000. Plates were incubated for 1 hour at room temperature on an orbital shaker. Plates were washed with TBST (0.1% TWEEN-20), and 100 µL of secondary antibody (Santa Cruz, goat anti-rabbit IgG-HRP, 1:2000, diluted in TBS) was added to each well. Plates were incubated for 1 hour at room temperature without agitation. Subsequently, plates were washed with TBST followed by ddH_2O, and 100 µL

of 3,3',5,5'-tetramethylbenzidine was added to each well. Plates were incubated in the dark for 10 minutes. The reaction was quenched with 2 N H_2SO_4, and the optical density was read at 450 nm using a SpectraMax 190 plate reader (Molecular Devices) with SoftMax Pro software (version 6.3). Total H3 and each of the three PTHMs were calculated relative to mixed calf histones based on a 4-parameter logistic standard curve. The %PTHM was calculated by dividing the PTHM measure by the total H3 measure. Samples from the same individual, but from different time points, were run on the same plate. %H3K79me2 values were normalized to the pooled blood sample to reduce potential batch effects (Ramanakumar et al. 2010). The inter-assay CV for %H3K79me2 was calculated from a subset of samples (n = 16) measured on two separate days. The intra- and inter-assay CVs, respectively, for each ELISA method were as follows: H3K36me2: 3.4% and 9.6%, H3K36me3: 4.9% and 11.9%, and H3K79me2: 7.1% and 7.0%. Since there were limited histone aliquots for the final assays, and since samples with poor reproducibility were excluded, final sample sizes for %H3K36me2 (n = 311) and %H3K36me3 (n = 300) were smaller than the final sample size for %H3K79me2 (n = 315).

Statistical Methods

Summary statistics were calculated for each variable (median (range) for continuous variables and % for categorical variables) in all participants and also separately by sex. Differences in continuous and categorical variables, between men and women and also between participants with and without %PTHM measures, were determined by Wilcoxon rank-sum and Chi-square tests, respectively. Transformations were applied to variables with skewed distributions to stabilize variances for parametric model assumptions and to reduce the influence of extreme values. A natural log-transformation (ln(X)) was applied to each of the predictors, bAs and uAs, which was adjusted for uCr (uAs_{Cr}); to the covariate BMI; and also to two of the

outcome variables, %H3K36me3, %H3K79me2. An inverse transformation (1/Y) was applied to the third outcome variable, %H3K36me2.

A generalized linear model, with an inverse-link function applied to the mean of (1/Y), was used to model the association between ln-bAs or ln-uAs_{Cr} and the harmonic mean of %H3K36me2. Associations between the predictors, ln-bAs and ln-uAs_{Cr}, and the outcomes, ln-%H3K36me3 and ln-%H3K79me2, were examined with linear models. Arsenic regression coefficients (β) in models for %H3K36me2 indicate the change in the harmonic mean of %H3K36me2 for a unit increase in ln-bAs or ln-uAs_{Cr}, controlling for other variables in the model, while those for %H3K36me3 and %H3K79me2 indicate the change in the mean of the ln-%PTHM for a unit increase in ln-bAs or ln-uAs_{Cr}, controlling for other variables in the model. Variables were considered potential confounders if they were correlated with arsenic exposure measures and the %PTHM in men or women and their addition to models changed arsenic exposure coefficients by > 10%. Therefore, we also present models adjusted for age, ln-BMI, education, and sex. To demonstrate the robustness of the associations between arsenic measures and %PTHMs, we also present analyses in supplemental materials showing adjustment for additional variables: ln-uCr, ln-plasma folate, and ln-plasma vitamin B12, and also cigarette and betel nut use (ever vs. never). All variables were included in models as continuous variables, except for sex and education; the latter was dichotomized (education > 5 years vs. ≤ 5 years), since many participants had 0 years of education. Models were also run separately by sex, and differences by sex were determined using the Wald test, which compares regression coefficients between models (Clogg et al. 1995).

We also present Spearman correlations, which remain the same with or without applying the specified transformations to variables, showing the relationships between arsenic measures

and %PTHMs to confirm that the directions of the associations are consistent with model-based results and to facilitate comparisons between %PTHMs.

Relationships between baseline and week 12 measures of each %PTHM were examined using Spearman correlations. The Wilcoxon signed-rank test was used to evaluate whether %PTHMs (untransformed) changed on average over a 12 week period; this was examined in a subset of participants (n = 60) in the placebo group (n = 56 for %H3K36me2 and %H3K79me2, n = 55 for %H3K36me3). We also examined the within-person changes in the %PTHMs separately by sex and tested for differences using the Wilcoxon rank-sum test.

A significance level of 0.05 was used for all statistical tests and regression models, which were performed with SAS (version 9.3, Cary, NC).

Results

General Characteristics, Arsenic Measures, and %PTHMs

General characteristics for the study participants are presented in **Table 1**. Participants were between 24 and 54 years old. Approximately 22.4% of the study participants had > 5 years of education. Blood arsenic concentrations ranged from 1.0 to 80.2 μg/L. Concentrations of uAs_{Cr} ranged from 35 to 2200 μg/g uCr. Compared with women, male study participants were older, had lower BMIs, and had higher uCr and bAs concentrations and lower uAs_{Cr} concentrations. Men were also more likely to have low plasma folate concentrations and to be cigarette smokers. Individuals in the placebo group with %PTHM measures (See **Supplemental Material, Table S1**) were very similar to the overall study population (Table 1). The only variables which differed were uCr concentrations and, consequently, uAs concentrations, which

were both lower in the placebo group participants. However, uAs_{Cr} concentrations were similar between groups.

FACT participants with %PTHM measures were generally comparable to FACT participants without %PTHM measures (See **Supplemental Material, Table S2**), although participants with %PTHM measures were slightly older, were more likely to have low folate, and had higher uCr and uAs concentrations (before adjustment for uCr).

Associations between Arsenic Exposure and %PTHMs

In the whole sample, neither ln-bAs nor ln-uAs_{Cr} was significantly associated with any of the %PTHMs (**Table 2**). However, ln-uAs_{Cr} was positively associated with %H3K36me2 in men both before ($\beta = 0.13$; 95% CI: 0.02, 0.24, $p = 0.02$) and after ($\beta = 0.12$; 95% CI: 0.01, 0.23, $p = 0.03$) adjusting for age, education, and ln-BMI. The association between ln-uAs_{Cr} and %H3K36me2 was in the opposite direction for women (covariate-adjusted $\beta = -0.05$; 95% CI: -0.12, 0.02, $p = 0.19$) and differed significantly from the corresponding estimate in men ($p = 0.01$) (Table 2). While not statistically significant, associations between ln-bAs and %H3K36me2 were similar to those for ln-uAs_{Cr}, with estimates that were positive in men and negative in women (p for difference between men and women = 0.08 for covariate-adjusted models). The patterns of associations according to sex were similar for ln-%H3K36me3. Since coefficients in models for %H3K36me2 represent changes in the harmonic mean of %H3K36me2, while coefficients in models for %H3K36me3 represent changes in the mean of ln-%H3K36me3, the magnitudes of the associations cannot be directly compared. However, the findings were consistent when examined by Spearman correlation, which does not require that variables be transformed and thus allows for more direct comparisons between %PTHMs (See **Supplemental Material, Table S3**). Although associations between ln-transformed arsenic measures and ln-%H3K36me3 were

not significant in either men or women, differences by sex were significant or suggestive (Table 2). Ln-transformed arsenic measures were not associated with ln-%H3K79me2 in men or women, and differences by sex were not significant (Table 2). Associations between arsenic measures and %PTHMs were very similar after additionally adjusting for ln-uCr, ln-plasma folate, ln-plasma B12, cigarette smoking status, and betel nut use (See **Supplemental Material, Table S4**).

Stability of %PTHMs after Reductions in Arsenic Exposure

Arsenic-removal water filter use for 12 weeks was associated with significant reductions in bAs (see Peters et al. 2015) and uAs_{Cr} ($p < 0.01$, Wilcoxon signed-rank test) in the placebo group. Summary statistics for within-person changes in %H3K36me2, %H3K36me3, and %H3K79me2 from baseline to week 12 for participants in the placebo group are presented in **Table 3**. Although for each of the three %PTHMs analyzed, baseline values were significantly correlated with values measured at week 12 (p-values from Spearman correlations < 0.01), the median change in %H3K36me2 from baseline to week 12 was negative (-0.15). Thus, on average %H3K36me2 declined over time ($p < 0.01$), although the interquartile range (IQR) for the within-person change (-0.43, 0.11) indicates that this mark did increase over time in at least 25% of participants. In sex-stratified analyses, %H3K36me2 was found to decrease on average among both men and women. However, the decline was only statistically significant among women ($p < 0.01$). %H3K36me3 did not change significantly during the 12 week period, but there was a suggestive decrease in %H3K79me2 (median within-person change: -0.05; IQR: -0.24, 0.04), $p = 0.07$). The within-person changes in %PTHMs did not differ significantly by sex (Table 3).

Discussion

In an adult population in Bangladesh, we examined associations between arsenic exposure and three %PTHMs (%H3K36me2, %H3K36me3, and %H3K79me2), which were selected because they are dysregulated in several types of cancer (Bernt et al. 2011; Duns et al. 2010; Fontebasso et al. 2013; Tamagawa et al. 2013; Zhang L et al. 2014) and are altered by arsenic *in vitro* (Zhou et al. 2008) or regulate 5-hmC (Huang et al. 2013; Williams et al. 2011). Percent 5-hmC has been shown to be altered by arsenic in male rats (Zhang J et al. 2014) and in humans in a sex-dependent manner (Niedzwiecki et al. 2015). We observed that arsenic was associated with higher levels of %H3K36me2, but only in men. Interestingly, the use of arsenic-removal water filters, which was associated with significant reductions in both bAs (Peters et al. 2015) and uAsCr, was also associated with significant reductions in %H3K36me2 in the full sample. However, in sex-stratified analyses, while we observed that %H3K36me2 declined in both men and women, this only achieved statistical significance among women. Given that there was no comparison group that did not receive arsenic-removal water filters due to ethical considerations, we cannot rule out the possibility that the decline in %H3K36me2 may have been caused by extrinsic factors. Additional studies will be needed to confirm the changes we observed in %H3K36me2 in association with arsenic removal. In cross-sectional analyses, arsenic exposure was not associated with %H3K36me3. Additionally, %H3K36me3 did not change over time, despite reductions in arsenic exposure. Thus, arsenic does not appear to alter %H3K36me3 in histones derived from PBMCs. Although in cross-sectional analyses arsenic exposure was not associated with %H3K79me2, which has been shown to regulate the expression of Tet1 (Huang et al. 2013; Williams et al. 2011), we did observe a suggestive (p =

0.07) decline in %H3K79me2 over time after individuals received arsenic-removal water filters; this needs to be confirmed in a larger sample.

Although neither %H3K36me3 nor %H3K79me2 changed significantly over the 12 week period, we cannot make definitive conclusions about the stability of these marks, since all participants received arsenic-removal water filters at baseline and were thus subject to an intervention; furthermore, these marks did vary over time in some participants. Little is known about the stability of %PTHMs in human PBMCs. One study by Zhang et al. examined the stability of PTHMs during adipogenesis and observed that while gene-specific levels of PTHMs were highly dynamic, global levels of PTHMs were remarkably stable (Zhang et al. 2012). However, since Zhang et al. used murine adipocyte cell lines, it is unclear if these findings are relevant to %PTHM stability in human PBMCs. One previous epidemiological study measured %PTHMs in PBMCs collected at three one-week intervals from 15 nickel refinery workers and 15 individuals who had not been exposed occupationally to nickel, and observed that the inter-individual variances in %PTHMs were much higher than the intra-individual variances, suggesting that %PTHMs are relatively stable over time in human PBMCs (Arita et al. 2012). However, since this was evaluated over a short duration, and in a small number of participants, this is an area that requires additional investigation.

Our study has several potential limitations. First, given the cross-sectional nature of some of the analyses, we need to consider the possibility of reverse causality. Since several previous experimental studies have shown that arsenic influences %PTHMs, it is unlikely that reverse causality would explain our findings. However, it is possible that %PTHMs influence the expression of genes involved in arsenic metabolism, such as the arsenic (+3 oxidation state) methyltransferase, which could thereby influence the excretion of arsenic, thus altering bAs and

uAs_{Cr} concentrations. Although residual confounding is another important consideration, the associations between arsenic measures and %PTHMs were quite robust, even after adjusting for additional covariates. Another important consideration for our study is the fact that %PTHMs were measured in human PBMCs, which consists of a mixed population of cell types. However, global DNA methylation levels, which are closely related to %PTHMs, have been shown to be very similar between blood cell types (reviewed in Smith and Meissner 2013). Additionally, a cross-sectional study of 63 male steel workers, which examined associations between inhalation exposure to occupational toxicants, including arsenic, and %PTHMs in total WBCs, evaluated the influence of cell type distribution on these associations, and observed that while adjusting for the proportion of granulocytes influenced their results, adjusting for other cell types did not have a major impact (Cantone et al. 2011). Since we measured %PTHMs in PBMCs, which do not include granulocytes, potential shifts in the proportion of granulocytes could not explain the associations we observed between arsenic and %PTHMs. However, we cannot rule out the possibility that alterations in the proportion of monocytes, natural killer cells, T cells, or B cells, or their subpopulations, may have affected our findings, and this is an area that merits additional investigation. Finally, an important limitation of our study is that the sample sizes for prospective analyses were small. Therefore, we may have had insufficient statistical power to formally examine sex differences in the influence of arsenic removal on %PTHMs.

Despite some of the limitations of this study, our findings support a previous experimental study in A549 cells, which examined the effects of arsenite (2.5 and 5 μM) on %H3K36me2 and %H3K36me3 (Zhou et al. 2008) and observed that arsenic decreased %H3K36me2 and increased %H3K36me3. Although we observed a positive association between uAs_{Cr} and %H3K36me2 in men and did not observe an association between uAs_{Cr} and

%H3K36me3, we studied a population exposed to arsenic-contaminated drinking water for years to decades, while Zhou et al. measured %PTHMs in a cell line that was exposed to arsenic for a 24 hour period. Furthermore, our study participants were healthy individuals, whereas A549 cells are alveolar basal epithelial cells derived from a male human lung tumor; arsenic may have distinct effects in different tissues and may also have differential effects in normal vs. cancerous cells. Additionally, *in vitro* studies are limited in that they cannot account for the numerous systemic differences associated with sex *in vivo*. Nevertheless, it is quite interesting that arsenic appears to target H3K36me2 in such diverse models.

Although the consequences of arsenic-induced increases in %H3K36me2 are currently unknown, H3K36me2 has been implicated in oncogenic programming (Kuo et al. 2011), and some of the enzymes responsible for regulating this mark, such as methyltransferase NSD2, are overexpressed in multiple cancers, including those caused by arsenic, such as bladder, lung, and skin cancers (Hudlebusch et al. 2011). A global increase in H3K36me2 leads to widespread increases in this mark across the genome, thereby altering its typical distribution (Popovic et al. 2014); this may have profound effects on both gene expression and genomic stability. For example, a global increase in H3K36me2 is associated with increased levels of H3K36me2 within gene bodies, which in turn is associated with increased expression of genes involved in oncogenic programming (Kuo et al. 2011).

Similar to our findings, several studies have observed sex-specific effects of arsenic on other epigenetic marks, such as DNA methylation (Broberg et al. 2014; Nohara et al. 2011; Pilsner et al. 2012), including our previous finding that arsenic exposure is associated with increased %5-mC and %5-hmC in men but not women (Niedzwiecki et al. 2015). Sex-specific effects of other environmental contaminants, such as cadmium and lead, on DNA methylation

have also been observed (Faulk et al. 2013; Kippler et al. 2013). Since PTHMs can direct DNA methylation patterns (Cedar and Bergman 2009), PTHMs may mediate the effects of environmental contaminants, such as arsenic, on DNA methylation marks. In addition to the sex-specific findings for uAs_{Cr} and %H3K36me2 reported here, our group previously observed sex-specific correlations between uAs_{Cr} and several other %PTHMs (Chervona et al. 2012). Additionally, Tyler et al. recently demonstrated that arsenic alters %PTHMs in a sex-dependent manner in the mouse brain (Tyler et al. 2015). Although the mechanisms are not fully understood, arsenic also altered the expression of corresponding histone modifying enzymes, including MLL and KDM5B, in a sex-dependent manner (Tyler et al. 2015). Many histone modifying enzymes have also been shown to interact with androgen receptor (Heemers and Tindall 2007), and some histone demethylases are dosage-sensitive regulators that are coded for by genes that reside on the Y chromosome and are highly conserved across mammalian species and broadly expressed across tissues and cell types (Bellott et al. 2014). Thus, both hormonal influences and genetic differences may contribute to the sex-specific effects of arsenic on %PTHMs.

For many arsenic-induced health outcomes, susceptibility differs by sex (NRC 2013). For example, men are more susceptible to developing arsenic-induced skin lesions (Ahsan et al. 2006b; Watanabe et al. 2001), and cancers of the skin, liver, and bladder (Chen and Wang 1990; Chen et al. 2003; Leonardi et al. 2012). In contrast, several studies have reported that early life exposure to arsenic is associated with impaired intellectual function and other developmental outcomes among female, but not male, children (Gardner et al. 2013; Hamadani et al. 2011; Saha et al. 2012). Additionally, in the United States the arsenic-associated risk for CVD was found to be higher among women (Moon et al. 2013). Animal studies have also demonstrated sex-specific

effects of arsenic for many outcomes. For example, female mice are more susceptible to arsenic-induced changes in locomotor activity (Bardullas et al. 2009) and are more likely to develop lung tumors as a result of prenatal exposure to arsenic, while males are more likely to develop liver and adrenal tumors (Waalkes et al. 2003).

Although some of the sex-specific effects of arsenic observed in human populations may be explained by gender-differences in co-exposures (e.g., cigarette smoking, UV exposure, and nutritional deficiencies), the dramatic sex differences observed in well-controlled animal studies of arsenic toxicity suggest that co-exposures are not solely responsible for these differences. One consideration is that women have a higher capacity to fully methylate inorganic arsenic to dimethyl arsenic species, which facilitates arsenic elimination in urine (Hopenhayn-Rich et al. 1996; Hsueh et al. 2003; Lindberg et al. 2007). This should generally reduce arsenic toxicity for women. However, some arsenic-related health outcomes preferentially afflict women, thus, there are likely other contributing factors. Epigenetic dysregulation has been implicated in the development of arsenic-induced health outcomes, including skin lesions and cancers of the skin and bladder (Chanda et al. 2006; Pilsner et al. 2009; Smeester et al. 2011; Wilhelm et al. 2010). Thus, epigenetic dysregulation may be one important mechanism contributing to the sex differences observed for multiple arsenic-related health outcomes. Previous studies examining the sex-specific effects of arsenic on epigenetics have focused on DNA methylation. However, this study and our previous study (Chervona et al. 2012) suggest that arsenic also induces sex-specific alterations in %PTHMs.

Conclusions

Our findings have two major implications that warrant further investigation. First, arsenic exposure was associated with %H3K36me2 in a sex-dependent manner in our study population

of adults in Bangladesh. While it is tempting to speculate that these findings may explain some of the observed sex differences in susceptibility to arsenic-induced diseases, the impact of %H3K36me2 and other %PTHMs on health outcomes will require further study. Secondly, the arsenic-associated increase in %H3K36me2 observed in men decreased, albeit non-significantly, after the use of arsenic-removal water filters. However, since we did not have a comparison group that did not receive water filters, and since %H3K36me2 decreased significantly in women, future studies will be needed to evaluate the effects of arsenic removal on %H3K36me2 and to investigate whether downstream effects of alterations in %PTHMs, such as changes in DNA methylation patterns, persist over time.

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Table 1. Baseline characteristics[a] of FACT participants with at least one %PTHM measure and complete information for variables included in regression models

Characteristic	Whole Sample (317)	Men (161)	Women (156)	*p*[b]
Age (years)	39 (24-54)	42 (25-54)	37 (24-54)	<0.01
BMI (kg/m^2)	19.3 (13.9-31.6)	18.7 (15.4-27.9)	20.0 (13.9-31.6)	<0.01
uCr (µg/L)	48 (6-252)	53 (6-252)	45 (6-233)	0.03
bAs (µg/L)	8.8 (1.0-80.2)	9.6 (2.5-52.0)	7.9 (1.0-80.2)	0.05
uAs (µg/L)	121 (11-1770)	123 (11-1770)	121 (11-1320)	0.67
uAs_{Cr} (µg/g uCr)	257 (35-2200)	242 (65-1480)	287 (35-2200)	0.03
%H3K36me2[c]	1.45 (0.68-6.87)	1.45 (0.68-4.00)	1.43 (1.00-6.87)	0.47
%H3K36me3[d]	1.61 (0.48-6.44)	1.57 (0.48-4.09)	1.62 (0.52-6.44)	0.18
%H3K79me2[e]	1.26 (0.29-9.46)	1.26 (0.29-9.46)	1.25 (0.29-9.41)	0.69
Folate < 9 nmol/L	74 (23.3)	46 (28.6)	28 (18.0)	0.03
B12 < 151 pmol/L	77 (24.3)	39 (24.2)	38 (24.4)	0.98
Ever Smoker	93 (29.3)	91 (56.5)	2 (1.3)	<0.01
Ever Betel	87 (27.4)	48 (29.8)	39 (25.0)	0.34
Education > 5 y	77 (22.4)	33 (20.5)	38 (24.4)	0.41

Abbreviations: bAs, blood arsenic; BMI, body mass index; FACT, Folic Acid and Creatine Trial; H3K36me2, di-methylation of lysine 36 of histone H3; H3K36me3, tri-methylation of lysine 36 of histone H3; H3K79me2, di-methylation of lysine 79 of histone H3; PTHM, post-translational histone modification; uAs, urinary arsenic; uAs_{Cr}, urinary arsenic adjusted for urinary creatinine; uCr, urinary creatinine

[a]Values are median (range) or n (%) for continuous and categorical variables, respectively

[b]Wilcoxon rank-sum or Chi-square test for difference between men and women in continuous and categorical variables, respectively

[c]n = 311 (Men: 158, Women: 153)

[d]n = 300 (Men: 153, Women: 147)

[e]n = 315 (Men: 161, Women: 154)

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Table 2. Estimated regression coefficients[a] and 95% confidence intervals for associations between baseline measures of arsenic exposure and %PTHMs in FACT participants

%PTHM	Arsenic Exposure	Whole Sample	Men	Women	*p*[b]
%H3K36me2[c]	bAs	0.02 (-0.05, 0.09)	0.12 (-0.00, 0.24)	-0.04 (-0.12, 0.04)	0.04
	bAs[d]	0.02 (-0.05, 0.09)	0.10 (-0.02, 0.22)	-0.03 (-0.11, 0.05)	0.08
	uAs_{Cr}	0.01 (-0.05, 0.08)	0.13 (0.02, 0.24)*	-0.05 (-0.12, 0.02)	<0.01
	uAs_{Cr}[d]	0.02 (-0.05, 0.08)	0.12 (0.01, 0.23)*	-0.05 (-0.12, 0.02)	0.01
%H3K36me3[e]	bAs	-0.03 (-0.10, 0.04)	0.06 (-0.04, 0.16)	-0.08 (-0.17, 0.01)	0.04
	bAs[d]	-0.02 (-0.09, 0.05)	0.05 (-0.04, 0.15)	-0.07 (-0.16, 0.02)	0.07
	uAs_{Cr}	-0.01 (-0.07, 0.05)	0.07 (-0.02, 0.16)	-0.06 (-0.14, 0.02)	0.04
	uAs_{Cr}[d]	0.00 (-0.06, 0.06)	0.07 (-0.02, 0.16)	-0.05 (-0.14, 0.03)	0.05
%H3K79me2[f]	bAs	0.04 (-0.05, 0.12)	0.04 (-0.09, 0.17)	0.03 (-0.08, 0.14)	0.91
	bAs[d]	0.03 (-0.05, 0.12)	0.04 (-0.09, 0.17)	0.04 (-0.08, 0.15)	0.95
	uAs_{Cr}	0.02 (-0.06, 0.09)	0.05 (-0.07, 0.17)	0.00 (-0.10, 0.10)	0.53
	uAs_{Cr}[d]	0.01 (-0.06, 0.09)	0.04 (-0.08, 0.17)	0.01 (-0.10, 0.11)	0.65

Abbreviations: bAs, blood arsenic; FACT, Folic Acid and Creatine Trial; H3K36me2, di-methylation of lysine 36 of histone H3; H3K36me3, tri-methylation of lysine 36 of histone H3; H3K79me2, di-methylation of lysine 79 of histone H3; PTHM, post-translational histone modification; uAs_{Cr}, urinary arsenic adjusted for urinary creatinine

[a]Estimated regression coefficients and 95% confidence intervals (β (CI)) from generalized linear models. Associations were examined between ln-bAs or ln-uAs_{Cr} in relation to each of the three %PTHMs. Coefficients from %H3K36me2 models indicate the change in the harmonic mean of %H3K36me2 for a unit increase in the ln-transformed arsenic measure, controlling for other covariates. Coefficients from %H3K36me3 and %H3K79me2 models indicate the change in the mean of the ln-%PTHM for a unit increase in the ln-transformed arsenic measure, controlling for other covariates.

[b]Wald test for sex difference

[c]Whole sample n = 311, Men n = 158, Women n = 153.

[d]Adjusted for age, education (dichotomized at 5 years), and ln-BMI. Whole sample analyses were also adjusted for sex.

[e]Whole sample n = 300, Men n = 153, Women n = 147.

[f]Whole sample n = 315, Men n = 161, Women n = 154.

*$p < 0.05$

Table 3. Within-person changes in %PTHMs from baseline to week 12 for FACT participants in the placebo group

%PTHM	**Whole Sample**			**Men**			**Women**			***p***[a]
	n	Median (IQR)	*p*[b]	n	Median (IQR)	*p*[b]	n	Median (IQR)	*p*[b]	
%H3K36me2	56	-0.15 (-0.43, 0.11)	<0.01	27	-0.07 (-0.44, 0.16)	0.14	29	-0.17 (-0.37, 0.04)	<0.01	0.62
%H3K36me3	55	0.02 (-0.23, 0.30)	0.61	28	0.05 (-0.23, 0.47)	0.35	27	0.02 (-0.22, 0.12)	0.93	0.35
%H3K79me2	56	-0.05 (-0.24, 0.04)	0.07	29	-0.03 (-0.28, 0.09)	0.36	27	-0.05 (-0.18, 0.04)	0.10	0.88

Abbreviations: FACT, Folic Acid and Creatine Trial; H3K36me2, di-methylation of lysine 36 of histone H3; H3K36me3, tri-methylation of lysine 36 of histone H3; H3K79me2, di-methylation of lysine 79 of histone H3; IQR, interquartile range; PTHM, post-translational histone modification

[a]Wilcoxon rank-sum test for difference between men and women in the within-person change for each %PTHM

[b]Wilcoxon signed-rank test for within-person change in %PTHM